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Lack of IL-6 augments inflammatory response but decreases vascular permeability in bacterial meningitis

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Summary

Interleukin (IL)-6 is a multifunctional cytokine with diverse actions and has been implicated in the pathophysiology of many neurological and inflammatory disorders. In this study, we investigated the role of IL-6 in pneumococcal meningitis. Cerebral infection in wild-type (WT) mice caused an increase in vascular permeability and intracranial pressure (ICP), which were significantly reduced in IL-6$^{-/-}$ mice. In contrast, meningitis in IL-6$^{-/-}$ mice was associated with a significant increase in CSF white blood cell count compared with infected WT mice, indicating an enhanced inflammatory response. Analysis of mRNA expression in the brain showed an increase in tumour necrosis factor (TNF)-α, IL-1β, and macrophage inflammatory protein 2 (MIP-2) levels, but decreased expression of granulocyte–macrophage colony-stimulating factor in infected IL-6$^{-/-}$ mice compared with infected WT controls. Similar results were obtained when rats challenged with pneumococci were systemically treated with neutralizing anti-IL-6 antibodies, resulting in an increased pleocytosis but at the same time a reduction of vascular permeability, brain oedema formation, and ICP, which was not accompanied by a downregulation of matrix metalloproteinases. Our data indicate that IL-6 plays an important anti-inflammatory role in bacterial meningitis by reducing leukocyte infiltration but contributes to the rise in intracranial pressure by increasing blood–brain barrier (BBB) permeability. These findings suggest that the migration of leukocytes across the BBB and the increase in vascular permeability are two independent processes during bacterial meningitis.

Keywords: chemokines; interleukin-6; meningitis; vascular permeability

Abbreviations: BBB = blood–brain barrier; EB = Evan's blue; GAPDH = glyceraldehyde-3-phosphate dehydrogenase; GM-CSF = granulocyte–macrophage colony-stimulating factor; i.c. = intracisternally; ICP = intracranial pressure; IL-1β = interleukin-1β; i.p. = intraperitoneally; MIP-2 = macrophage inflammatory protein 2; MMP = matrix metalloproteinase; PBS = phosphate-buffered saline; PMN = polymorphonuclear neutrophils; TNF-α = tumour necrosis factor α; WBC = white blood cell; WT = wild type

Introduction

Bacterial meningitis is associated with a high morbidity and mortality (Quagliarello and Scheld, 1992). The reasons for the unfavourable clinical outcome include cerebrovascular alterations, breakdown of the blood–brain barrier (BBB), formation of vasogenic brain oedema and increase in intracranial pressure (ICP) (Pfister *et al*., 1992). Several mediators of the pathophysiological pathway contributing to these intracranial complications have been identified, including nitric oxide (Koedel *et al*., 1995), reactive oxygen species (Koedel and Pfister, 1999) and matrix metalloproteinases (MMPs) (Paul *et al*., 1998). Moreover, experimental and clinical studies suggest that cytokines including tumour necrosis factor (TNF)-α (Nadal *et al*., 1989) and interleukin (IL)-1β (Quagliarello *et al*., 1991), and chemokines such as

macrophage inflammatory protein 2 (MIP-2) (Diab *et al*., 1999), also play an important role in the pathophysiology of bacterial meningitis.

IL-6 is a multifunctional cytokine with diverse actions, e.g. regulation of inflammation including the induction of the acute phase reaction, immune response and cellular differentiation (Gadient and Otten, 1997). Increased cerebral expression of IL-6 has been demonstrated in many CNS diseases, such as HIV encephalopathy, multiple sclerosis and Alzheimer's disease (Gadient and Otten, 1997). High levels of IL-6 were also detected in the CSF of patients with viral and bacterial meningitis (Houssiau *et al*., 1988; Matsuzono *et al*., 1995). In a previous study using a rat model of pneumococcal meningitis we found elevated concentrations of IL-6 in the CSF 6 h after challenge (Koedel *et al*., 1996). However, the precise role of IL-6 in the pathophysiology of inflammatory and CNS diseases has not been elucidated. On one hand, IL-6 was reported to have beneficial effects by reducing neuronal damage in cerebral ischaemia (Loddick *et al*., 1998), preventing cartilage destruction during experimental arthritis (van de Loo *et al*., 1997), and acting as an anti-inflammatory cytokine in endotoxemia (Xing *et al*., 1998). On the other hand, transgenic mice lacking the *IL-6* gene were resistant to the pathophysiological alterations in experimental autoimmune encephalomyelitis (Eugster *et al*., 1998), and mice overexpressing IL-6 in astrocytes showed neuropathological abnormalities with a breakdown of the BBB (Brett *et al*., 1995), suggesting a detrimental role of IL-6 in neurological diseases associated with increased BBB permeability.

The aim of this study was to investigate the impact of IL-6 on the pathophysiology of pneumococcal meningitis using IL-$6^{-/-}$ mice as well as neutralizing anti-IL-6 antibodies in a rat model. We demonstrate that during bacterial meningitis IL-6 acts as an anti-inflammatory cytokine by suppressing the transmigration of leukocytes into the CSF space, but plays a major role in the increase of vascular permeability, causing brain oedema and increase in ICP, indicating that these are two independent processes.

Material and methods

Mouse model of pneumococcal meningitis

All of the experiments were approved by the government of Upper Bavaria. Sixteen male and female mice were used in this study. C57BL/6 mice were purchased from Charles River (Sulzfeld, Germany). The IL-$6^{-/-}$ mice, backcrossed into C57BL/6 mice, were a kind gift from Hoffmann-La Roche Ltd (Basel, Switzerland). For induction of bacterial meningitis the mice were short-term anesthetized with halothan (Hoechst AG, Frankfurt/M, Germany) and 15 µl 10^7 colony forming units/ml of *Streptococcus pneumoniae* strain type 3 were injected transcutaneously into the cisterna magna. Control mice received 15 µl of phosphate-buffered saline (PBS) intracisternally (i.c.), instead of pneumococci. The mice were housed individually in cages, allowed to wake up naturally, and fed with a standard diet and water *ad libitum*.

After 24 h the mice were anesthetized intraperitoneally (i.p.) with 100 mg/kg chloralhydrate, and the left femoral vein was cannulated for fluid substitution and the administration of Evan's Blue (EB) dye. The body temperature of the mice was kept constant at 37.5 ± 0.5°C using a rectal thermometer-controlled heating pad. A burr hole was made at the occipital bone and a catheter was inserted into the cisterna magna for ICP monitoring and collection of CSF samples to determine CSF white blood cell (WBC) count at the end of the experiment.

Determination of BBB permeability in the mouse model

BBB permeability was determined as described previously by Gijbels *et al*. (1994). Mice were injected intravenously with 0.1 ml of 1% (w/v) EB. After 1 h the brains were perfused with 10 ml of ice-cold PBS and removed. The brains were weighed and tissue samples were extracted for 3 days in formamide (5 µl/mg tissue) and the extract centrifuged for 5 min at 500 *g*. EB concentration in the supernantants was determined by measuring the absorbance at 650 nm. The BBB permeability index was calculated by dividing the value for each sample by the mean value of the control animals.

Mouse intracerebral injection and determination of BBB disruption

Determination of the effect of IL-6 on cerebral vascular permeability was performed as previously described (Paul *et al*., 2001). Recombinant human IL-6 (Biosource, Cammarillo, CA, USA) was injected stereotactically into the left frontal lobe (1.5 mm left and 0.5 mm rostral from bregma, 2.5 mm in depth from the dura) of C57BL/6 mice. PBS was injected into the right hemisphere as a control. The animals received 100 µl of a 2% EB solution intravenously 15 min after IL-6 injection. After additional 45 min, the brains were perfused with PBS and removed. Whole-mount direct EB fluorescence was observed using confocal microscopy of fresh infixed brains.

Detection of mouse mRNA levels by RT-PCR

Frozen brains from IL-$6^{-/-}$ (n = 3) and wild-type (WT) mice (n = 3) were cut with a cryostat. Total RNA was prepared from frozen sections containing lateral ventricules and hippocampal tissue with Trizol-LS-reagent (Gibco-BRL, Gaithersburg, MD, USA). Oligo(dt)-primed cDNA was prepared from 5 µg total RNA using Superscript II reverse transcriptase (Gibco-BRL) as recommended by the manufacturer. Specific primers were designed for granulocyte–macrophage colony-stimulating factor (GM-CSF) (sense 5′-TTC CTG GGC ATT GTG GTC T-3′; antisense 5′-TGG ATT

CAG AGC TGG CCT GG-3′), MIP-2 (sense 5′-AGT TTG CCT TGA CCC TGA AGC C-3′; antisense 5′-TGG GTG GGA TGT AGC TAG TTC C-3′), β-actin (sense 5′-GGA CTC CTA TGT GGG TGA CGA GG-3′; antisense 5′-GGG AGA GCA ATA GCC CTC GTA AGA T-3′) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (sense 5′-CAT CAC CAT CTT CCA GGA GCG-3′; antisense 5′-GAG GGG CCA TCC ACA GTC TTC-3′).

Of the cDNA product, 1.0 µl (corresponding to 125 ng RNA) was used for PCR in a reaction mix (40 µl) consisting of 0.18 U Taq DNA polymerase (Amplitaq Gold; Perkin Elmer, Foster City, CA, USA), 1 mM of each primer, 0.2 mM dNTP mix, 2.5 mM $MgCl_2$ and 4.0 µl of 10× PCR buffer. Negative controls with RNA before reverse transcription were performed to ensure cDNA amplification. PCR was performed in an Eppendorf thermocycler using the following profile: denaturation at 94°C for 30 s, annealing at individual temperatures (61–66°C) for 30 s and elongation at 72°C for 1 min 30 s. The 25 cycles (GAPDH), 35 cycles (MIP-2) and 40 cycles (GM-CSF) used were in the linear range of amplification. PCR products were seperated on a 1.7% agarose gel and stained with ethidium bromide. Photographs were scanned and analysed by densitometry. PCR was carried out in duplicate and the mean of product density was expressed relative to GAPDH and β-actin controls, respectively. Specifity of PCR products was confirmed by DNA sequencing. The mRNA expression ratio between infected and uninfected mice was calculated by dividing the value for each sample of an infected mouse by the mean value of the uninfected mice. All experiments were performed in duplicate.

Rat model of pneumococcal meningitis

A modification of a well-established rat model of pneumococcal meningitis was used for the experiments which was previously described in detail (Koedel and Pfister, 1997). Thirty-one adult male Wistar rats (Charles River) were used in this study. For induction of bacterial meningitis the rats were short-termed anesthetized with halothan (Hoechst AG) and 150 µl 10^7 colony forming units/ml of *Streptococcus pneumoniae* strain type 3 were injected transcutanously into the cisterna magna. Control rats received 150 µl PBS instead of pneumococci. The rats were housed individually in cages, allowed to wake up naturally, and fed with a standard diet and water *ad libitum*.

Twenty-four hours after infection the rats were anesthetized i.p. with 100 mg/kg thiopental (Trapanal, Byk Gulden, Germany), tracheotomized and artificially ventilated with a small animal ventilator (Model AP-10; Effenberger, Pfaffing, Germany). A catheter was inserted into the left femoral artery for continuous monitoring of mean arterial blood pressure and for analysis of p_aCO_2, p_aO_2, and pH with a blood gas analyser (IL 1304; Instrumentation Laboratory, Kirchheim, Germany). The left femoral vein was cannulated for fluid substitution and EB administration. The body temperature of the rats was kept constant at 37.5 ± 0.5°C using a rectal thermometer-controlled heating pad. A burr hole was made at the occipital bone and a catheter was inserted into the cisterna magna for continuous ICP monitoring, collection of CSF samples and determination of CSF WBC at the end of the experiment.

Determination of BBB permeability in the rat model

For visualization of the BBB permeability during meningitis rats were injected intravenously with 1ml of 1% (w/v) EB (Sigma, Taufkirchen, Germany). After 1 h the brains were perfused with 100 ml of ice-cold PBS, removed and stored at –80°C for further histological examinations. EB was observed under green fluorescence microscopy (excitation filter 545 nm, barrier filter 590 nm) and appeared red.

Rat IL-6 enyzme immunoassay

IL-6 concentration in the CSF were determined by a commercially available immunoassay kit (Laboserv, Staufenberg, Germany) that is specific for rat IL-6 and does not cross-react with rat IL-1β, rat IL-10 and rat TNF-α. The minimum detectable dose of rat IL-6 was 31 pg/ml. CSF samples with enzyme levels above the detection limit were appropriately dissolved in dilutent buffer.

Anti-IL-6 antibody

For neutralization of IL-6 bioactivity *in vivo* a polyclonal goat anti-murine IL-6 antibody (R & D Systems GmbH, Wiesbaden, Germany; <10 ng of endotoxin/mg of protein) was diluted to a concentration of 500 µg/kg body weight in 0.5 ml of PBS and injected i.p. The ability of this antibody to react with rat IL-6 and to neutralize IL-6 bioactivity was demonstrated *in vivo* and *in vitro* in previous studies (Gennari and Alexander, 1995; Shanley *et al*., 1997).

Rat mmunohistochemistry

Sections (10 µm thick) were fixed with 100% ethanol. Immunohistochemistry was performed according to the instructions using the Vectastain® ABC kit (Alexis Deutschland GmbH, Grünberg, Germany). A polyclonal goat anti-murine IL-6 antibody (see above) served as the primary antibody at a dilution of 20 µg/ml at 4°C overnight. Endogenous peroxidase activity was suppressed by incubating the sections with 0.3% H_2O_2 in methanol for 30 min prior to the primary antibody. The stained slides were counterstained with Mayer's hemalum solution (Merck Diagnostic, Darmstadt, Germany) and examined by light microscopy.

Competitive RT–PCR of rat MMP mRNA

For quantitation of rat MMP mRNA levels, PCR using a multi-competitior DNA standard was performed as described previously (Kieseier *et al*., 1999). Briefly, primer pairs for collagenase-3, matrilysin, gelatinase A, gelatinase B, stromelysin-1, -2 and -3, and β-actin were used according to the sequences published elsewhere. Poly(A^+) RNA was extracted from fozen brain specimens ($n = 3$) and used as a template for cDNA snthesis using AMV reverse transcriptase (Promega, Madison, WI, USA). Three-fold serial dilutions of competitive standard cDNA were combined with a fixed amount of sample cDNA, and PCR was performed in 50 µl reactions containing 200 µM dNTP, 50 pmol of sense and antisense MMP primers, 1 U Taq DNA polymerase (Perkin Elmer, Branchburg, NJ, USA) and 1 µCi [α-^{32}P]dCTP (Amersham, Braunschweig, Germany). Ten microlitres of the reaction products were eletrophoresed on a 6% polyacrylamide gel, exposed to appropiate screens, and the uptake of [α-^{32}P]dCTP within each individual PCR product was determined using a PhosphorImaging system (Storm 360; Molecular Dynamics, Krefeld, Germany). Levels of MMP mRNA were determined by plotting the ratio of sample cDNA to standard DNA against the standard dilution using a double logarithmic scale.

Experimental groups

Seven experimental groups were investigated: group 1, six rats were injected i.c. with PBS (controls); group 2, 11 rats were injected i.c. with pneumococci; group 3, six rats were injected i.c. with pneumococci and treated with 500 µg/kg anti-IL-6 antibody i.p. at the time of infection; group 4, eight rats were injected i.c. with pneumococci and treated with 500 µg/kg anti-IL-6 antibody i.p. 6 h after infection; group 5, three WT mice were injected i.c. with PBS (controls); group 6, five WT mice were injected i.c. with pneumococci; group 7, eight IL-6$^{-/-}$ mice were injected i.c.with pneumococci.

Statistical analysis

All values are expressed as mean ± SEM. Data sets of group 1 and 2, and 6 and 7 (BBB permeability index) were compared using the unpaired Student's *t*-test. Data sets of group 2, 3 and 4, and 5, 6 and 7 (CSF WBC count) were compared using one-way ANOVA (analysis of variance) and Scheffe's test. Differences were considered significant at $P < 0.05$.

Results

IL-6 is an anti-inflammatory cytokine in meningitis

I.c. injection of pneumococci caused a significant increase of CSF-IL-6 levels in rats after 24 h ($P < 0.05$) compared with uninfected controls (Fig. 1A). Immunohistochemistry was performed on brain sections after pneumococcal challenge and in controls, respectively. During meningitis IL-6 was localized predominantly in the leptomeningeal space surrounding pial vessels, but also in the underlying cortical parenchyma (Fig. 1C). No IL-6 could be detected in controls with this method (Fig. 1B).

Pneumococcal challenge in C57Bl/6 WT mice significantly increased CSF WBC count after 24 h ($P < 0.05$) compared with uninfected controls (Fig. 2A). Similar results were obtained in rats challenged i.c. with pneumococci or PBS (controls), respectively (Fig. 2B). In IL-6$^{-/-}$ mice, i.c. injection of pneumococci caused a significant increase in CSF WBC count, which was almost three times higher than in infected WT mice ($P < 0.05$) (Fig. 2A). Likewise, rats treated systemically with neutralizing antibodies against IL-6 showed an increased inflammatory response. Irrespective of the time of administration (15 min before or 6 h after infection), antibody treatment augmented meningitis-induced CSF pleocytosis by ~35% (not significant), showing a trend to an increased inflammatory response (Fig. 2B). These data demonstrate that IL-6 acts as an anti-inflammatory cytokine in bacterial meningitis suppressing the migration of leukocytes into the CSF space.

Cerebral TNF-α, IL-1β and MIP-2 expression are increased in the absence of IL-6 during bacterial meningitis

To determine the reasons for the increased leukocyte count in the infected IL-6$^{-/-}$ mice we investigated the changes of cerebral expression of TNF-α, IL-1β, GM-CSF and MIP-2 using RT–PCR 24 h after injection of pneumococci and PBS, respectively. No expression of TNF-α and IL-1β mRNA was found in uninfected WT and IL-6$^{-/-}$ mice (data not shown), whereas profound differences in the basal mRNA content of GM-CSF and MIP-2 were detected between the two strains (Fig. 3A). GM-CSF expression in IL-6 $^{-/-}$ mice was approximately twice that in WT mice, whereas MIP-2 mRNA was almost absent (Fig. 3A).

Meningitis caused the induction of cerebral mRNA expression of all investigated cytokines/chemokines in WT as well as in IL-6$^{-/-}$ mice after 24 h. Both, TNF-α and IL-1β mRNA expression were increased in infected WT and IL-6$^{-/-}$ mice (Fig. 3B). However, the elevation of the mRNA content was more pronounced in IL-6$^{-/-}$ mice compared with infected WT mice, indicating that the lack of IL-6 promotes the expression these pro-inflammatory cytokines (Fig. 3B). Similar results were found among the chemokines. Since the basal gene expression of GM-CSF and MIP-2 was different in both strains, the ratio of mRNA levels in infected versus uninfected mice was calculated to assess the impact of IL-6 on mRNA expression. GM-CSF mRNA content in the brain was more than eight times higher in infected WT mice compared with WT controls, whereas in IL-6$^{-/-}$ mice GM-CSF expression was increased only more than four times (Fig. 3C). These data indicate that GM-CSF mRNA

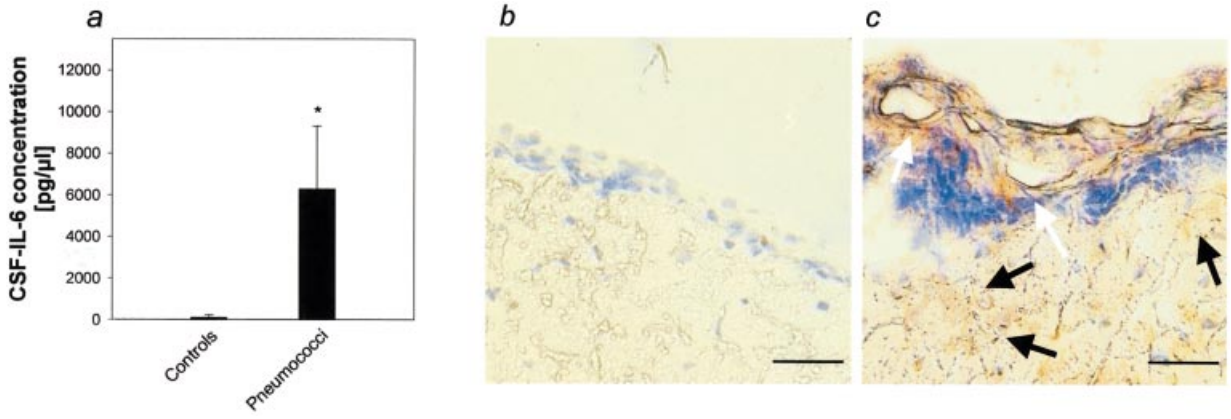

Fig. 1 IL-6 expression during bacterial meningitis. (**A**) I.c. injection of pneumococci significantly increased IL-6 levels in the CSF of rats after 24 h. Localization of IL-6 by immunohistochemistry: (**B**) no IL-6 expression could be detected in controls; (**C**) in contrast, strong staining for IL-6 was evident in infected rats in the leptomeningeal space surrounding pial vessels (white arrows) and in the underlying cortical parenchyma (black arrows). $^{*}P < 0.05$ compared with controls. Data are expressed as mean + SEM. Bar = 50 µm.

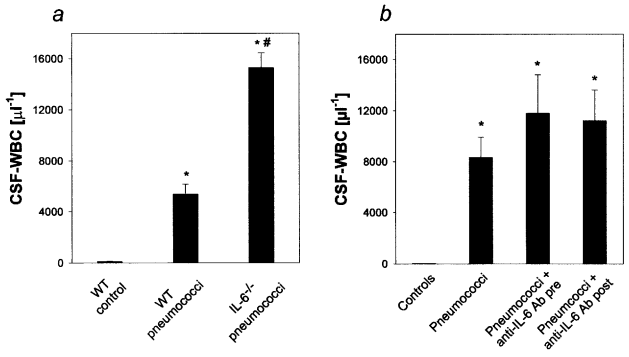

Fig. 2 IL-6 is an anti-inflammatory cytokine in bacterial meningitis. (**A**) CSF WBC count was significantly higher in IL-6 deficient mice (IL-6$^{-/-}$) compared with WT mice 24 h after infection. (**B**) Similar results were obtained in rats challenged i.c. with pneumococci and treated with neutralizing antibodies against IL-6. Irrespective of the time of administration (15 min before or 6 h after infection) antibody treatment augmented meningitis-induced CSF pleocytosis (not significant). $^{*}P < 0.05$ compared with controls; $^{\#}P < 0.05$ compared with infected WT. Data are expressed as mean + SEM.

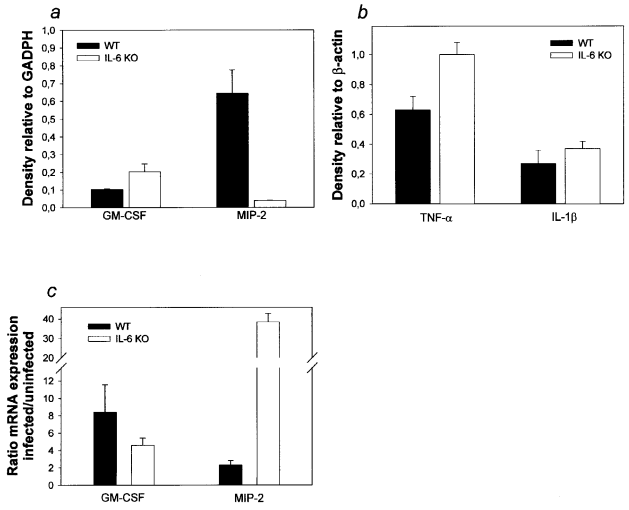

Fig. 3 Expression of TNF-α, IL-1β, GM-CSF and MIP-2 mRNA in brain specimens. (**A**) Differences in the basal mRNA content of GM-CSF and MIP-2 were found between uninfected WT and IL-6$^{-/-}$ mice. GM-CSF mRNA expression in uninfected IL-6$^{-/-}$ mice was increased approximately twice compared with control WT mice, whereas MIP-2 was almost absent in IL-6$^{-/-}$ mice. (**B**) Meningitis induced the expression of TNF-α and IL-1β mRNA in WT mice, which was further increased in IL-6$^{-/-}$ mice, indicating an enhanced inflammatory response. No message for these cytokines was found in uninfected controls in either strains. (**C**) To assess the impact of IL-6 on gene expression during meningitis the ratio of mRNA content in infected versus uninfected mice was calculated. MIP-2 expression was 16-fold increased in IL-6$^{-/-}$ mice compared with WT mice, whereas GM-CSF message was almost 50% reduced in infected IL-6$^{-/-}$ mice compared with infected WT mice. Data are expressed as mean + SEM.

upregulation is attenuated in the absence of IL-6 during bacterial meningitis.

In contrast, cerebral MIP-2 expression was 38 times higher in infected IL-6$^{-/-}$ mice, compared with a two-fold increase in infected WT mice (Fig. 3C). These results suggest that IL-6 downregulates MIP-2 mRNA expression during bacterial meningitis.

IL-6 increases BBB permeability and ICP

BBB permeability in mice was assessed by measuring the permeability index using EB as a marker for extravasation of serum proteins. Bacterial meningitis in control mice caused leakage of cerebral blood vessels as demonstrated by an increase in the permeability index and a significant increase in ICP 24 h after infection ($P < 0.05$) (Fig. 4A and B). In infected IL-6$^{-/-}$ mice, however, the rise in ICP was significantly attenuated to almost basal levels ($P < 0.05$), and the

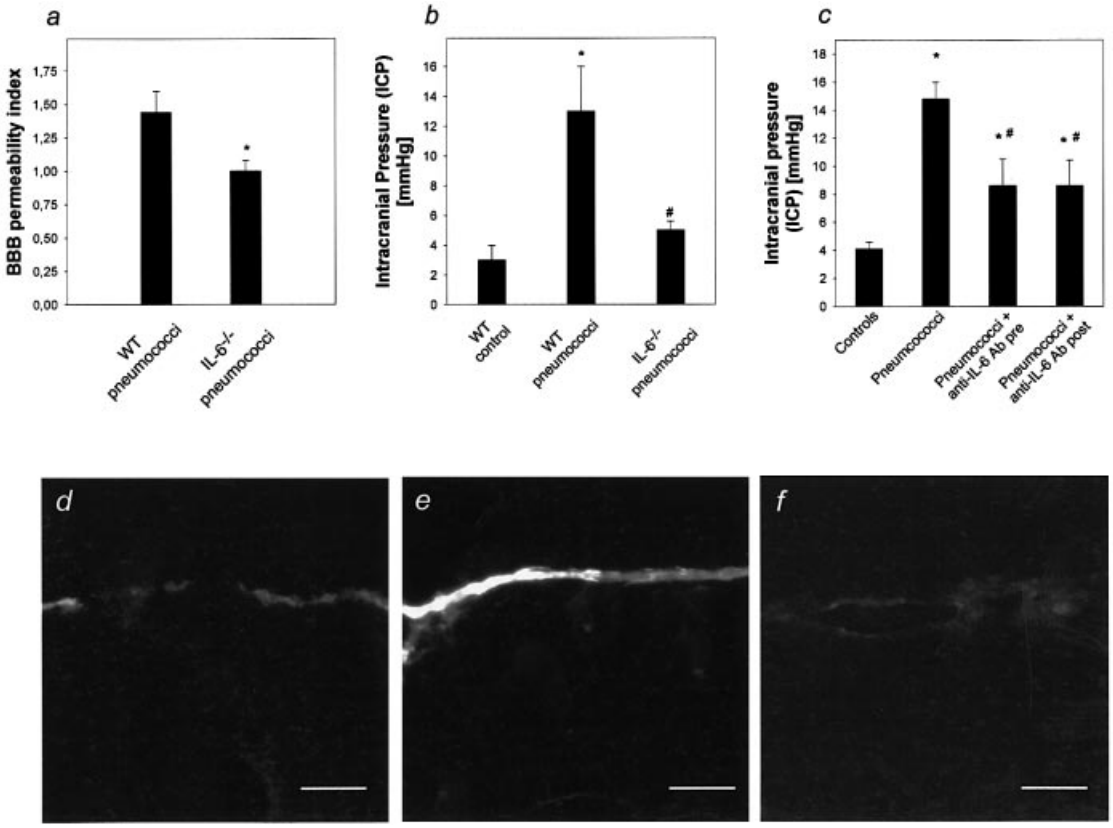

Fig. 4 IL-6 increases BBB permeability during pneumococcal meningitis. (**A**) I.c. injection of pneumococci increased BBB permeability index in WT mice which was significantly reduced in infected IL-6$^{-/-}$ mice ($^{*}P < 0.05$ compared with infected WT). (**B**) Disruption of the BBB in infected WT mice was associated with a significant increase in ICP. However, in infected IL-6$^{-/-}$ mice the ICP was significantly reduced to almost normal levels ($^{*}P < 0.05$ compared with WT controls; $^{\#}P < 0.05$ compared with infected WT mice). (**C**) Similar results were obtained when rats, challenged with pneumococci, were treated with neutralizing anti-IL-6 antibodies. Irrespective of the time of administration (before or 6 h after infection), anti-IL-6 antibody treatment significantly reduced the pneumococci-induced increase in ICP ($^{*}P < 0.05$ compared with uninfected controls; $^{\#}P < 0.05$ compared with infected, untreated rats), which was accompanied by a diminished BBB permeability as assessed by EB extravasation. (**D**) Only minor EB leakage was detectable in the leptomeningeal space and no dye was found in the brain parenchyma demonstrating an intact BBB in control rats. (**E**) Meningitis caused leakage of EB into the subarachnoidal space and the underlying cerebral parenchyma 24 h after infection. (**F**) Extravasation of EB was attenuated in anti-IL-6 antibody-treated rats, showing only weak fluorescence in the subarachniodal space and no fluorescence in the brain parenchyma. Data are expressed as mean + SEM. Bar = 50 μm.

permeability index was significantly decreased ($P < 0.05$), indicating that vascular leak was prevented in these mice (Fig. 4A and B).

These findings were confirmed in rats treated with neutralizing anti-IL-6 antibodies. BBB permeability was assessed using fluorescence microscopy to detect cerebral extravasation of systemically administered EB. Only minor EB leakage was detectable in the leptomeningeal space and no dye was found in the brain parenchyma, demonstrating an intact BBB in control rats (Fig. 4D). Bacterial meningitis caused exudation of EB into the subarachnoidal space and the underlying cerebral parenchyma 24 h after infection indicating the disruption of the BBB (Fig. 4E). Extravasation of EB was attenuated in anti-IL-6 antibody-treated rats, showing only weak fluorescence in the subarachniodal space and no fluorescence in the brain parenchyma (Fig. 4F). Meningitis-induced breakdown of the BBB was associated with a significant increase in ICP in untreated rats ($P < 0.05$) (Fig. 4C). Treatment with anti-IL-6 antibodies before or 6 h after infection significantly reduced the increase in ICP to the same extent ($P < 0.05$) (Fig. 4C).

To confirm the direct impact of IL-6 on cerebral vessel permeability, recombinant human IL-6 was injected stereotactically into the cortex of WT mice. Extravasation of intravenously injected EB was evident 1 h after recombinant human IL-6 application, indicating increased BBB permeability at the site of injection (Fig. 5A). No EB fluorescence could be detected in the contralateral PBS-injected control hemisphere (Fig. 5B). Together with the findings described above, these results demonstrate that IL-6 plays an important role in the breakdown and sustained opening of the BBB.

Expression of MMPs is not mediated by IL-6

In previous studies we have shown that expression of MMPs, especially MMP-9, is associated with disruption of BBB in

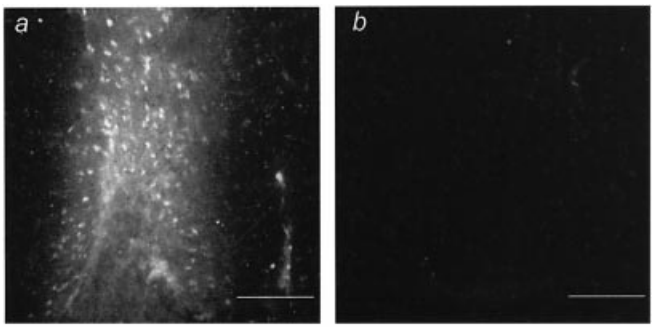

Fig. 5 Effect of IL-6 on BBB permeability. (**A**) Direct injection of recombinant human IL-6 into the cerebral cortex of WT mice increased vessel permeability as assessed by extravasation of systemically administered EB. (**B**) No EB leakage was detected in the contralateral side, which was injected with PBS. Bar = 100 μm.

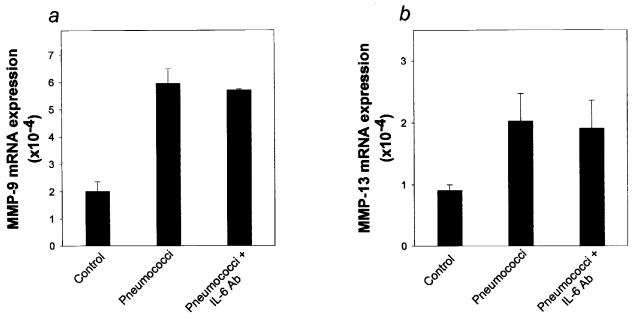

Fig. 6 Expression of MMP-9 and MMP-13 mRNA in rats treated with anti-IL-6 antibodies. Cerebral infection of rats with pneumococci caused an increase in (**A**) MMP-9 and (**B**) MMP-13 mRNA expression compared with uninfected controls. No reduction in mRNA levels were found in infected rats pre-treated with anti-IL-6 antibodies. Data are expressed as mean + SEM.

bacterial meningitis (Paul *et al*., 1998; Kieseier *et al*., 1999). Therefore, we investigated the effect of IL-6 on the meningitis-induced expression of different MMPs in rat brains using competitive quantitative RT–PCR. Similar to the results shown in a previous study (Kieseier *et al*., 1999), we found constitutive expression of stromelysin-3 (MMP-11), gelatinase A (MMP-2) and matrilysin (MMP-7) at low levels, and no expression of stromelysin-2 (MMP-10). None of these MMPs was upregulated 24 h after infection (data not shown). Expression of stromelysin-1 (MMP-3), which was previously proven to be increased 6 and 9 h after infection (Kieseier *et al*., 1999), was no longer upregulated after 24 h (data not shown). However, cerebral mRNA expression of gelatinase B (MMP-9) (Fig. 6A) and collagenase-3 (MMP-13) (Fig. 6B) was increased in infected rats at 24 h. Pre-treatment with neutralizing anti-IL-6 antibody did not influence the expression of these MMPs (Fig. 6A and B) or the activity of MMP-9 as assessed by zymography (data not shown). These data indicate that IL-6 does not mediate the expression or activity of MMPs during bacterial meningitis.

Discussion

A major complication of bacterial meningitis is an increased permeability of the cerebral microvessels, resulting in vasogenic brain oedema and a rise in ICP (Pfister *et al*., 1999). The breakdown of the BBB was always considered to be the consequence of the invasion of leukocytes releasing cytokines, reactive oxygen species and proteases, leading to an increase in vascular permeability. Here we show that IL-6 contributes substantially to the disruption of the BBB, but at the same time acts as an anti-inflammatory cytokine in bacterial meningitis. These findings demonstrate that the migration of leukocytes across the BBB and the increase in vascular permeability are two independent processes.

Bacterial meningitis in IL-6$^{-/-}$ mice caused a three-fold increase in CSF WBC count compared with infected WT mice, which was associated with increased TNF-α, IL-1β and MIP-2 mRNA expression. TNF-α and IL-1β are well-characterized pro-inflammatory cytokines in bacterial meningitis and both can initiate meningeal inflammation (Ramilo *et al*., 1990). Our study shows that IL-6 attenuates mRNA expression of both cytokines, indicating that IL-6 acts as an anti-inflammatory cytokine. Similar results were found by others who documented increased TNF-α and IL-1β protein expression in IL-6$^{-/-}$ mice during acute inflammation (van der Poll *et al*., 1997; Xing *et al*., 1998), affirming the anti-inflammatory nature of IL-6. MIP-2, which parallels the action of IL-8 in humans (Broxmeyer *et al*., 1996), is a member of the CXC family of chemokines and a potent chemoattractant of polymorphonuclear neutrophils (PMN) (Lahrtz *et al*., 1998). MIP-2 was shown to be an important mediator of PMN influx in many inflammatory diseases such as ocular bacterial infection and urinary tract infection (Hang *et al*., 1999; Kernacki *et al*., 2000). The importance of MIP-2 in attracting PMN during bacterial meningitis was demonstrated in *in vitro* (Spanaus *et al*., 1997) and *in vivo* (Diab *et al*., 1999) studies, which proved that neutralization of MIP-2 attenuated neutrophil chemotaxis. Our study is in accordance with results obtained by others (Xing *et al*., 1998; Hurst *et al*., 2001) who showed the regulatory role of IL-6 during acute inflammation where MIP-2 mRNA and protein expression was increased in IL-6$^{-/-}$ mice compared with WT mice, resulting in an enhanced neutrophilic response. It was also shown that IL-6 can attenuate the TNF-α- and IL-1β-mediated release of IL-8 (Hurst *et al*., 2001), the human homologue of MIP-2, which might explain the increase of MIP-2 in infected IL-6$^{-/-}$ mice. The elevated number of leukocytes cannot be accounted for by the prolongation of the lifetime of these cells, since the absence of IL-6 does not affect the percentage of apoptotic neutrophils during inflammation, as shown by others (Brach *et al*., 1992; Xing *et al*., 1998). The highly reduced mRNA expression of MIP-2 in uninfected IL-6$^{-/-}$ compared with WT controls found in our study also suggests that the basal expression of this pro-inflammatory chemokine is compensatorily downregulated to counteract the absence of the anti-inflammatory IL-6.

On the other hand, expression of GM-CSF in infected IL-6$^{-/-}$ mice was also reduced compared with infected WT mice. GM-CSF can directly act on PMN by modulating the surface expression of adhesion molecules and increasing neutrophil adherence to the endothelium (Arnaout *et al*., 1986). It was shown that GM-CSF can induce the expression of IL-6 in monocytes and microglia (Suzumura *et al*., 1996) and that *vice versa* IL-6 can inhibit *GM-CSF* gene expression (Kremlev *et al*., 1998), presenting a negative feedback system which might explain the fact that the basal GM-CSF mRNA level in uninfected IL-6$^{-/-}$ mice was more than twice that in WT controls. Therefore, it was surprising that the increase in GM-CSF mRNA expression was attenuated in infected IL-6$^{-/-}$ mice, compared with infected WT mice. However, similar results were observed in IL-6$^{-/-}$ mice after a focal cryo brain injury, which showed reduced GM-CSF protein expression compared with injured WT controls (Penkowa *et al*., 1999). Despite the reduced GM-CSF expression in the absence of IL-6 we found higher CSF WBC counts in infected IL-6$^{-/-}$ mice compared with infected WT mice, confirming the results of a previous study which demonstrated that neutrophilic infiltration in lungs of GM-CSF-deficient mice is increased after pulmonary streptococcal infection (LeVine *et al*., 1999). It is unclear whether the increased inflammatory response is directly related to the reduced GM-CSF expression or is an indirect effect of GM-CSF on other chemokines. For example, it was shown that MIP-2 levels are elevated in GM-CSF$^{-/-}$ mice with pulmonary streptococcal infection compared with infected WT mice (LeVine *et al*., 1999), indicating that GM-CSF can downregulate MIP-2 expression. Therefore, we suggest that decreased GM-CSF expression in IL-6$^{-/-}$ mice also might have contributed to the enhanced CSF pleocytosis during bacterial meningitis by increasing MIP-2 expression.

Despite the fact that leukocyte recruitment was enhanced in infected IL-6$^{-/-}$ mice and IL-6 antibody-treated rats, the disruption of the BBB was significantly reduced, suggesting that transmigration of leukocytes and increase in vascular permeability are two independent processes. In this study we demonstrated the direct impact of IL-6 on cerebral vessel permeability by injecting the cytokine directly into the brain. There are several other studies which showed that IL-6 influences the integrity of the BBB. For example, IL-6 was demonstrated to reduce the transendothelial electrical resistance (de Vries *et al*., 1996) and to induce changes in the morphology and permeability of endothelial cells in an *in vitro* model of the BBB (Duchini *et al*., 1996). Moreover, the systemic administration of IL-6 increased the permeability of the BBB in rats (Saija *et al*., 1995), and transgenic mice overexpressing IL-6 in astrocytes showed extensive extravasation of horseradish peroxidase indicating an open BBB (Brett *et al*., 1995).

Previously, we demonstrated that MMPs, especially MMP-9, are involved in the disruption of the BBB and oedema formation during bacterial meningitis (Paul *et al*., 1998; Kieseier *et al*., 1999). Therefore, we reasoned that IL-6 might regulate MMP expression or activity. However, in this study no reduction in the expression of MMP-9 and MMP-13 or activity of MMP-9 was found, despite the significant decrease in vascular permeability in rats treated with anti-IL-6 antibodies. These results suggest that MMPs may not be directly involved in the disruption of the BBB. It seems that MMPs mediate the migration of leukocytes rather than directly disturb endothelial barrier function, and that the beneficial effect of MMP-inhibition on brain oedema formation might be secondary to its anti-inflammatory effect. These results are in accordance with *in vitro* studies showing that increased endothelial permeability is a not necessary consequence of PMN migration (Huang *et al*., 1988; Lennon *et al*., 1998). Our findings rather suggest that IL-6 is released by migrating leukocytes and/or activated resident cells, which in turn increases cerebral vascular permeability contributing to the formation of brain oedema during bacterial meningitis.

Taken together, the results of this study show that IL-6 increases BBB permeability during bacterial meningitis, causing the formation of brain oedema, but inhibits leukocyte recruitment possibly by suppressing the expression of TNF-α, IL-1β and MIP-2. Dissecting the downstream signalling pathways of IL-6 affecting leukocyte migration or vascular permeability will be helpful to better understand the pathophysiology of bacterial meningitis and other inflammatory diseases.

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